Indonesian Journal of Agricultural Science 2(2) 2001: 31-36

CRYOPRESERVATION OF TRUE-SEED AND EMBRYO OF MAIZE AND SOYBEAN FOR LONG-TERM STORAGE

Enny Sudarmonowati[a], I. Fitryatmi[b], and S. Sadjad[b]

[a]*Research Centre for Biotechnology-LIPI, Jalan Raya Bogor km 46, Cibinong 16911, Indonesia*
[b]*Seed Science and Technology Study Program, Faculty of Agriculture, Bogor Agricultural University, Jalan Raya Darmaga 122, PO Box 220, Bogor 16680, Indonesia*

ABSTRACT

Study on cryopreservation of Indonesian local cultivars and improved varieties of maize and soybean has never been done. This method may be used for long-term preservation of seeds of maize and soybean. In this study, the method was applied to maize and soybean, Arjuna and Wilis respectively, as a model for preserving germplasm of ortodox seeds. Whole seeds and excised embryos of both varieties were subjected to two methods of cryopreservation, i.e., two-stage cooling and rapid freezing with or without 15% dimethyl sulfoxide (DMSO) as cryoprotectant solution prior to immersion in liquid nitrogen (-196°C). Results indicated that there was no significant difference between the use of DMSO for both species in terms of viability, although pretreatment in DMSO was slightly reduced the percentage of viability of both species. Slow freezing to -30°C prior to immersion in the liquid nitrogen could give as high as 76.67% and 51.67% surviving whole seeds of maize and soybean, respectively. Preserving excised embryos of maize in the liquid nitrogen using either slow or rapid freezing significantly reduced the percentage of viability from 20-76.67% to 5-18.33% (four folds) depending on treatments applied. Results also showed that one day or 15 minutes of immersion of samples in the liquid nitrogen gave rise to similar values of viability of maize and soybean, i.e., 20-60% and 20-51.67%, respectively depending on treatments applied. These results implied that for long-term storage of maize and soybean seeds as they could survive at the rate of 76.67% and 51.67% respectively, the seed can be treated by prefreezing to -30°C without the presence of DMSO prior to immersion in liquid nitrogen.

[*Keywords*: maize; soybean; seed storage; embryo preservation]

INTRODUCTION

Loss of plant germplasm collection or genetic resources due to either natural events or human exploitation has dramatically increased during the past few years. Efforts to conserve genetic diversity for breeding purpose or securing the availability of planting materials are therefore important.

Cryopreservation, i.e., preservation in liquid nitrogen (-196°C) offers the best method for long-term preservation of plant germplasm without genetic change. During the cryopreservation, the biochemical and biological activities of the plant are ceased (O'Hara and Henshaw, 1980). There are several cryopreservation techniques such as two-stage cooling or widely known as slow cooling, rapid freezing, vitrification and encapsulation-dehydration methods. These techniques have been applied to mostly annual crops in temperate regions. The success of the first two methods has been very limited as it seems to be genotype-specific one, while the last two methods have been recently developed and seems to be more appropriate for preserving recalcitrant seeds. The rapid freezing method is more suitable for smaller size ortodox seeds compared to larger seeds. The slow cooling, on the other hand, requires simple combination of cryoprotectants, or even one type of cryoprotectant such as dimethyl sulfoxide (DMSO) or glycerol at a moderate concentration. The results of both slow cooling and rapid freezing have been reported in *Hevea brasiliensis* (Engelmann *et al*., 1997) and cassava seeds (Mumford and Grout, 1978) which showed that slow cooling was better than rapid freezing.

Factors affecting the success of cryopreservation are the type of cell and tissue used, genotype of the plant, water content, type and concentration of cryoprotectant solution, length of exposure in cryoprotectant solution, cooling rate, thawing procedure, and regeneration media. All factors play a critical role in cryopreservation work in all plant species although certain factors play a greater role than the others which usually related with plant genotypes.

Arjuna and Wilis are improved maize and soybean varieties respectively, released by the Government of Indonesia. Both varieties are characterized by their high yield and fast growing characters. As the high-yielding varieties are not usually resistant to certain plant pests and diseases, planting these varieties in a large scale at the same place and the same time might

lead to the extinction of the varieties due to the attack of plant pests and diseases or the occurrence of natural catastrophe. Conservation through storage of either seeds or embryos of improved varieties, endemic or wild species which possess important traits for breeding or improvement of plant species, will prevent the loss of genetic resources. Breeding efforts which certainly require plants possessing superior characters to obtain better traits which could provide the need and natural pressures in the future are of importance.

Both maize which belongs to the family of Poaceae (Subandi *et al*., 1988) and soybean which belongs to the family of Leguminoceae seeds are considered as ortodox seeds. These seeds are smaller in size than the recalcitrant ones, consequently they contain lower water content and germination time is longer which is possible to be stored for certain period even at room temperature. However, the longest period of storage at room temperature or at 4^{o}C (in a cold room or a refrigerator) might be up to 1-3 years, depending on the species, which is con-sidered as short-term storage.

Cryopreservation offers a long-term storage up to unlimited time without the loss of genetic stability. Among ortodox seeds, certain species are more difficult to be stored than the others, for example soybean seeds are more difficult to be preserved (Sadjad, 1993). The use of both species was mainly based on consideration to explore the possibility to conduct *ex situ* conservation of agricultural crops in the laboratory as conservation in the field requires larger area and is exposed to unexpected natural catastrophe such as disease, long drought, and flood.

The aims of the study were to compare the effect of different freezing temperatures (-5, -10, -30, and -196^{o}C) and terminal temperatures (-5, -10, and -30^{o}C) prior to storage in liquid nitrogen and freezing procedures (slow or rapid cooling) in long-term preservation of whole seeds or excised embryos of soybean and maize as a model of ortodox seeds of agricultural crops.

MATERIALS AND METHODS

Seed Source

Seeds of both maize Arjuna variety and soybean Wilis variety were obtained from research field of Bogor Agricultural University in Darmaga, Bogor, West Java. Planting design and maintenance of plants in the field were made to secure the purity of the plants and to optimize the growth

Drying Procedure

As soon as maize and soybean were harvested, they were then sun-dried to achieve water content of 16% for maize and 12% for soybean measured using a water content apparatus. The corn was then removed from the cobs and the beans were removed from the pods prior to further drying using a seed-drying machine to obtain stable water content for storage until used for planting.

Embryo Excision

Embryos were excised by removing cotyledons from soybean seeds and endosperm from corn seeds using a sterile scalpel. As excision of maize embroys was difficult due to its position that are strongly intact to the endosperm, the seeds were previously soaked in water.

Treatment of Cryoprotectant

A half-batch of both whole seeds (120) and excised embryos (120) of each maize and soybean were soaked in 15% DMSO solution for 2 hours, while the other half were prepared for freezing without pre-treatment in DMSO. Samples of whole seeds and embryos of untreated and treated with DMSO were then wrapped in aluminum foil, each containing 20 samples, then each were placed in cryotubes (Nunc.). Each tube of those treated with DMSO was given one drop of 15% DMSO before the tube was caped. All tubes were then placed in the aluminum canes and frozen directly in liquid nitrogen or subjected to different terminal temperatures (-5, -10, and -30^{o}C) prior to immersion in liquid nitrogen.

Freezing Procedure

Samples were immersed in a methanol bath of the programmable freezer (ET-1, Japan) set to a cooling rate of 0.5^{o}C minute^{-1}. Unless otherwise stated, samples were subjected to three different terminal freezing temperatures, i.e., -5, -10 or -30^{o}C. After the temperature reached -5^{o}C, all samples were subjected to ice formation induction by touching the base of the tubes with long forceps previously dipped in liquid nitrogen. The ice formation could be observed by the formation of white patch at the base of the tubes. Samples subjected to -5^{o}C were taken out at this condition and then directly plunged in liquid

nitrogen, while those subjected to -10°C and -30°C were put back to the programmable freezer for further freezing up to these terminal temperatures before immersion in liquid nitrogen for 15 minutes. For comparison, some samples that have been frozen to -5°C and -10°C were kept in liquid nitrogen for 24 hours (one day).

Thawing

All samples were taken out from the liquid nitrogen tank and tested their viability. Samples were first unwrapped then were thawed by soaking them in sterile distilled water at laboratory room temperature (+25°C) for 30 minutes. The procedure used was a modified procedure for embryogenic callus of cassava (Sudarmonowati and Henshaw, 1992). This slow thawing procedure was also meant for washing the trace of DMSO from the samples.

Germination Test

All samples including the control treatments, i.e., untreated with DMSO and unfrozen were placed on moistened layers of special paper made from rice straw placed on the same size of a piece of plastic. Samples were wrapped by rolling the paper and the plastic carefully, then placed in a germinator set at 25°C and 90% humidity until observation. Viable samples which were indicated by germination were first observed at 5 days after planting for maize while 3 days for soybean. Second observation was conducted after 7 days and 5 days for maize and soybean, respectively. Seeds or excised embryos were considered viable when their embryos grew longer and emerged out of the seeds.

Research Design and Statistical Analysis

Complete Random Design was applied and statistical analysis of F values was carried out using significant test at 5%.

RESULTS AND DISCUSSION

Effect of DMSO on Survival of the Frozen Seeds

Pretreatment of 15% DMSO before freezing at -5, -10, -30 or -196°C did not seem to benefit frozen whole seeds as it did not increase the viability percentage of maize and soybean in all experiments tried including the use of excised embryos and longer exposure in liquid nitrogen, i.e., one day exposure. Viability of the unfrozen seeds of maize and soybean soaked in 15% DMSO for 2 hours decreased from 93.33% to 81.67% and from 98.33% to 95%, respectively, while that of slow-frozen ones reduced more than half of the viable samples of both commodities (Table 1). However, the pretreatment could slightly increase (13.33;20.00% became 15.83;26.67%) the survival of frozen whole seeds of maize when the seeds were subjected to rapid freezing (Tables 1 and 2). This might be because seeds which were subjected to rapid freezing as they were encountered to a shock as compared to slow freezing, required appropriate dehydration and cryoprotectant solution to prevent the formation of intracellular ice crystal. Figures 1 and 2 show the germination of soybean seeds unpretreated with DMSO and maize seeds pretreated with DMSO after freezing at -5°C and -30°C and storage in liquid nitrogen.

Unlike maize, the reduction of the viability percentage of seeds that were subjected to freezing regardless terminal temperatures and freezing procedures, was noticeable although it was not significantly different. This suggests that soybean seeds seem to be more tolerant to low temperatures than that of maize. The results also indicated that 15% DMSO used for soaking seeds of both maize and soybean for 2 hours was slightly harmful or toxic to the seeds as the percentage viability of those treated with DMSO but not frozen was reduced as compared to control even when the seeds were not pretreated and not frozen, i.e., 81.67% vs 93.33% and 95% vs 98.33% for maize and soybean, respectively (Table 1). Lower percentages of seeds treated with DMSO and subjected to low temperatures also suggest that the penetration of DMSO was presumably not yet optimal to fully protect the formation of ice crystal when the seeds were subjected to freezing.

DMSO at the concentration of 5-15% has been reported beneficial for organ or tissue cryopreservation of several plant species such as embryogenic callus of oil palm (Engelmann and Dereuddre,1988) and shoot tips of potato (Bajaj, 1985a). Somatic embryos of cassava treated with 15% DMSO gave the highest frequency of survival (50%) compared with lower concentrations such as 5% and 10% which produced only up to 8.3% survival (Sudarmonowati and Henshaw, 1992). According to Merryman and Williams (1982), DMSO is able to penetrate into the cells which prevents them from over losses of cell liquid.

Table 1. Effect of pretreatment of 15% DMSO, terminal freezing temperatures, and freezing procedures on the percentage of viability of maize and soybean seeds.

Species	Treatments	Viability (%)
Maize	Control (-D, -F)	93.33a
	+D, -F	81.67ab
	-D, -5°C	78.33ab
	+D, -5°C	55.00bc
	-D, -5°C, LN	26.67cde
	+D, -5°C, LN	25.00cde
	-D, -30°C	45.83cd
	+D, -30°C	16.67de
	-D, -30°C, LN	40.83cde
	+D, -30°C, LN	24.17cde
	-D, LN	13.33e
	+D, LN	15.83de
Soybean	Control (-D, -F)	98.33a
	+D, -F	95.00a
	-D, -5°C	92.50a
	+D, -5°C	84.17ab
	-D, -5°C, LN	58.33abc
	+D, -5°C, LN	22.50cde
	-D, -30°C	50.00bcd
	+D, -30°C	15.83de
	-D, -30°C, LN	51.67bcd
	+D, -30°C, LN	16.67de
	-D, LN	36.67cde
	+D, LN	7.50e

+D = with DMSO, -D = without DMSO, LN = liquid nitrogen, -F = without freezing

Values with the same letters in the same column are not significantly different (at 5%).

Effect of Terminal Temperatures Prior to Immersion in Liquid Nitrogen on the Viability of Seeds

Maize seeds were more susceptible or vulnerable than soybean seeds to low temperature during the first-step cooling prior to the immersion in liquid nitrogen. Slow freezing to -30°C at a rate of 0.5°C minute^{-1} has increased, although it was not significant, the viability percentage of maize whole seeds as compared to that to -5°C (40.83% vs 26.67%), which indicates that the terminal temperature is critical, while that of soybean was not affected by terminal temperature very much (Table 1). This suggests that different species behave differently towards freezing. Slow freezing to -30°C, which freezing effect is more stable than -5 or -10°C, is beneficial for certain species. Terminal temperatures at -30 or -40°C have been proven beneficial to increase the survival of plant species such as potato (Bajaj, 1985a), oil palm (Engelmann and Dereuddre, 1988), and carrot (Withers, 1979).

Fig. 1. Germination of unpretreated DMSO-soybean seeds and after freezing at -5°C or 30°C and storage in liquid nitrogen. Note that seeds stored in liquid nitrogen (LN) could grow normally compared to control.

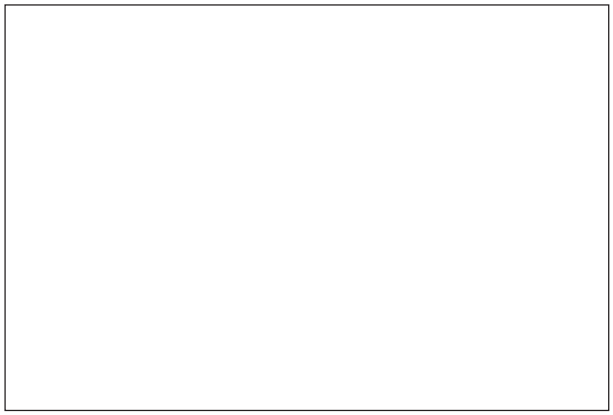

Fig. 2. Germination of pretreated DMSO-maize seeds after slow freezing at -5°C or -30°C and storage in liquid nitrogen. Note that seeds slow freezed to -30°C followed by immersion in liquid nitrogen could grow normally and vigorously (code -30+D) and that one seed frozen to -5°C prior to liquid nitrogen exposure showed abnormal growth (see arrow).

Interaction Between Seed Parts, Terminal Temperature, and Freezing on the Viability of Seeds

The viability percentage of excised embryos of maize was much lower than that of whole seeds. The differences of the values were very significant for those subjected to either slow cooling to any terminal temperature (-5 or -30°C) prior to immersion in liquid nitrogen or direct plunge in liquid nitrogen (Table 2). This result was not in accordance with that obtained with *Azadirachta indica* (Berjack and Dumet, 1996) and with peach (de Boucad *et al.*,1996). They have proven the logic that smaller size of plant organs subjected to cryopreservation is the better in terms

Table 2. Effect of interaction between seed parts, terminal temperatures, and freezing procedures on the viability of maize seeds.

Treatments	Viability (%)	
	Whole seeds	Excised embryos
Control (-D, -F)	100.00a	86.67abc
+D, -F	93.33ab	70.00abcde
-D, -5°C	86.67abc	70.00abcde
+D, -5°C	75.00abcd	35.00bcde
-D, -5°C, LN	50.00abcde	3.33f
+D, -5°C, LN	43.33abcde	6.67e
-D, -30°C	73.33abcd	18.33de
+D, -30°C	20.00de	13.33de
-D, -30°C, LN	76.67abcd	5.00f
+D, -30°C, LN	43.33abcde	5.00f
-D, LN	20.00de	6.67ef
+D, LN	26.67cde	5.00f

+D = with DMSO, - D = without DMSO, LN = liquid nitrogen, -F = without freezing

Values with the same letters in the same column or row are not significantly different (at 5%).

of viability as smaller organ contains smaller water which make the dehydration easier. The result suggests that excision has presumably caused serious injury to the embryos which made them more succeptible to freezing which caused significant reduction from 50-76.67% to 3.33-5% (Table 2). The injury occurred during excision has even caused a significant reduction in the percentage of germination of control ones which were not frozen (data not shown). The injury was occurred as the excision was difficult due to the position of the embryos which was slightly covered by the endosperm while the whole seeds were dried which made excision even more difficult. Soaking the seeds in sterile water prior to excision was not really ease the excision.

Effect of Freezing on the Viability of Seeds

Rapid freezing by placing samples directly in liquid nitrogen without prior slow cooling to certain temperature caused the viability of both whole seeds of maize and soybean untreated with DMSO reduced from 76.67% to 20% and from 58.33% to 46.67%, respectively (Tables 2 and 3). Seeds of maize which were not treated with DMSO and directly plunged in liquid nitrogen have produced significantly lower percentage of viability as compared to those subjected to two-stage cooling at -5 and -30°C, i.e., 13.33% vs 26.67% and 40,83% (Table 1). Similarly, viability percentage of soybean directly immersed and that of two-stage cooling was 36.67% vs 58.33% and 51,67% (Table 1). This means that treatment for hardening samples prior to immersion in liquid nitrogen is crucial for viability of both plant species tested. Sakai and Sugawara (1973) also showed that slow cooling and cold hardening prior to cryopreservation improved the survival of frozen tissues of *Populus americana*. Similar result was also reported for *Dianthus caryophyllus* (Seibert and Wetherbee, 1977).

In some cases, rapid freezing could also lead to loss of morphogenesis competence as reported by Sudarmonowati and Henshaw (1992), in work with somatic embryos of cassava which caused the frozen tissues only produced friable callus due to the loss of embryogenic competence. An optimum procedure developed for each species to obtain a high survival rate without losing certain competency such as embryogenic and regeneration competences needs to be studied.

Effect of Length of Exposure in Liquid Nitrogen on the Viability of Whole Seeds

Period of exposure of samples in liquid nitrogen caused different results on the viability percentage of whole seeds of maize and soybean. The percentages of viability of maize seeds were almost similar between that exposed for 15 minutes and in 24 hours in liquid nitrogen, that of slow cooled to -5°C prior to immersion in liquid nitrogen even showed a higher value for 24-hour exposure. Similar response was shown by the whole seeds of soybean. However, storage in liquid nitrogen (24 hours) resulted in 51.67% and 60% surviving seeds of soybean and maize, respectively (Table 3). This result was in accordance with many investigators that 15 minutes were sufficient for assessing viability after storage in liquid nitrogen as tissues or organs that could withstand liquid nitrogen (-196°C) for 10-15 minutes could be stored for unlimited time. Bajaj (1985b) in work with potato and cassava shoot tips has obtained high percentage of viability after storage in liquid nitrogen for 4 years. Without a proper preconditioning such as dehydration to certain level of water content and treatment in cryoprotectant solution, exposure for 10-15 minutes could kill all organs, tissues or cells as shown in many researches such as cryopreservation of embryonic axis and whole seeds of *Shorea* spp. and *Nephelium lappaceum* (data were not published).

Table 3. Effect of length of liquid nitrogen exposure on the viability of maize and soybean seeds.

Species	Treatments	Viability (%)	
		15 min	24 h
Maize	-D, -5°C, LN	50.00	60.00
	+D, -5°C, LN	43.33	46.67
	-D, -10°C, LN	70.00	60.00
	+D, -10°C, LN	43.33	36.67
	-D, LN	20.00	20.00
	+D, LN	26.67	23.33
Soybean	-D, -5°C, LN	58.33	51.67
	+D, -5°C, LN	18.33	23.33
	-D, -10°C, LN	45.00	40.00
	+D, -10°C, LN	18.33	20.00
	-D, LN	46.67	30.00
	+D, LN	15.00	23.33

+D = with DMSO, -D = without DMSO, LN = liquid nitrogen

CONCLUSION

The ability of both seeds of maize and soybean to recover after storage in liquid nitrogen although the percentage was still up to 76.67% and 58.33%, respectively using two-stage cooling indicated that these species could be stored for a long-term basis. There is a possibility to increase the survival by improving sterilization of organs, pretreatment procedure including type and concentration of cryoprotectant solution, and thawing procedure.

Viability percentage of frozen whole seeds of maize either by two-stage cooling or direct/rapid freezing was significantly higher than excised embryos. Therefore, the use of whole seeds of maize is recommended for future storage as a model of ortodox seeds of food crops.

Simplification of cryopreservation of both maize and soybean seeds is also possible by optimization of direct or rapid freezing without pretreatment in DMSO as survival rate of 20-46.67%, respectively, could be obtained. A more appropriate drying procedure to obtain optimum water content and thawing procedure are, therefore, required to increase the survival.

REFERENCES

Bajaj, Y.P.S. 1985a. Cryopreservation of embryos. p. 227-242. *In* K.K. Kartha (Ed.). Cryopreservation of Plant Cells and Organs. CRC Press Inc., Boca Raton, Florida, USA.

Bajaj, Y.P.S. 1985b. Cryopreservation of germplasm of potato (*Solanum tuberosum* L.) and cassava (*Manihot esculenta* Crantz): viability of excised meristems cryopreserved up to four years. Indian J. Exp. Biol. 23: 285-287.

Berjack, P. and D. Dumet. 1996. Cryopreservation of seeds and isolated embryonic axes (*Azadirachta indica*). Cryo-Letters 17: 99-104.

de Boucad, Marie-Therese, B. Helliot, and M. Brison. 1996. Desiccation and cryopreservation of embryonic axes of peach. Cryo-Letters 17: 379-390.

Engelmann, F. and J. Dereuddre. 1988. Cryopreservation of oil palm somatic embryos: importance of the freezing process. Cryo-Letters 9: 220-235.

Engelmann, F., M. Lartaud, N. Chabrillange, M.P. Carron, and H. Etienne. 1997. Cryopreservation of embryogenic calluses of two commercial clones of *Hevea brasiliensis*. Cryo-Letters 18: 107-116.

Merryman, H.T. and R.J. Williams. 1982. Basic principles of freezing injury to plant cells: natural tolerance and approaches to cryopreservation. p. 13-47. *In* K.K. Kartha (Ed.). Cryopreservation of Plant Cells and Organs. CRC Press Inc., Boca Raton, Florida, USA.

Mumford, P.M. and M.W.W. Grout. 1978. Germination and liquid nitrogen storage of cassava seeds. Ann. Bot. 42: 255-257.

O'Hara, J.F. and G.G. Henshaw. 1980. Cryopreservation and strategies for plant germplasm conservation. Cryo-Letters 1: 261-266.

Sadjad, S. 1993. Dari benih ke benih. PT Gramedia, Jakarta. 143 hlm.

Sakai, A. and Y. Sugawara. 1973. Survival of poplar callus at super low temperatures after cold acclimation. Plant Cell Physiol. 14: 1201-1204.

Seibert, M. and P.J. Wetherbee. 1977. Increased survival and differentiation of frozen herbaceous plant organ cultures through cold treatment. Plant Physiol. 59: 1043-1046.

Subandi, M. Syam, and A. Widjono. 1988. Jagung. Pusat Penelitian dan Pengembangan Tanaman Pangan, Bogor. 423 hlm.

Sudarmonowati, E. and G.G. Henshaw. 1992. Cryopreservation of cassava embryogenic callus and somatic embryos. Proceedings of Seminar on Results of Research and Development of Biotechnology, Bogor, 14-16 September 1992. Research and Development Centre for Biotechnology, Bogor.

Withers, L.A. 1979. Freeze preservation of somatic embryos and clonal plantlets of carrot (*Daucus carota*). Plant Physiol. 63: 460.